Pergamon

Bioorganic & Medicinal Chemistry 10 (2002) 1639–1646

BIOORGANIC & MEDICINAL CHEMISTRY

Novel Tn Antigen-Containing Neoglycopeptides: Synthesis and Evaluation as Anti Tumor Vaccines

Laura Cipolla,* Maria Rescigno,† Antonella Leone,‡ Francesco Peri, Barbara La Ferla and Francesco Nicotra*

Department of Biotechnology and Biosciences, Università degli Studi di Milano-Bicocca, P.za della Scienza 2, 20126 Milan, Italy

Received 25 September 2001; accepted 17 December 2001

Abstract—The fully unprotected α-*C*-glycosyl analogue of *N*-acetylgalactosamine **9** was conjugated by a non-natural oxime bond to the segment peptides $^{328-340}$OVA and $^{327-339}$OVA, affording neoglycopeptides **1**–**2** and **3**, having one or two sugar units, respectively. The three neoglycopeptides were tested in vitro in an antigen presentation assay as antitumor vaccines. Neoglycopeptides **1**–**3** could be presented to and recognized by the T cell receptor; neoglycopeptide **3**, bearing two B-epitopes, was presented to the TCR with higher efficiency, compared to neoglycopeptide **2**, having only one B-epitope.

Introduction

The Tn antigen (GalNAcα-*O*-Ser/Thr) is a carbohydrate epitope overexpressed in various human carcinomas[1] of epithelial origin, which comprise by far the largest group of malignant tumors. In most of these tumors the Tn antigen, one of the blood group precursor antigens, is found exposed on the external surface membranes. In all other tissues, the Tn antigen is masked and not accessible to the immune system. Moreover the extent of Tn expression often correlates with carcinoma differentiation and aggressiveness. Thus, carcinomas have antigens recognized as foreign by the host immune system and that could be exploited to induce an anticancer immune response.[2] The ultimate goal in the design of an antitumor vaccine is to develop vaccines specific for a tumor antigen (active specific immunotherapy, ASI)[3] using well characterized chemically synthesized antigens as immunogens. Unfortunately, synthetic antigens are, usually, poorly or not at all immunogenic, being low molecular weight compounds or self-antigens.[4] In general, a good vaccine has to induce an immunologic memory of long duration, that means that it must stimulate memory B and T lymphocytes.[5] This argument is also true for vaccines against tumors since they do not have to provoke only an immediate and strong humoral response, such to eliminate the tumor cells present in the organism, but also a long-lasting protection against the formation of micrometastases.

The design of an antitumor vaccine is not that simple: in order to elicit an IgG humoral response, B cells have to internalize the antigen through their B cell receptor and present antigenic peptides on MHC II molecules for T cell help. Thus, a bivalent antigen constituted by a saccharidic B epitope and a T epitope is desirable. To do so, saccharidic tumor antigens have been coupled to suitable protein carriers,[6] including serum albumin from different sources, as chicken serum albumin (CSA),[7] bovine serum albumin (BSA),[8] human serum albumine (HSA),[9] keyhole limpet hemocyanin (KLH),[8b,10] or ovine submaxillary mucin (OSM).[11] The latter was used in a very elegant study by Singhal et al. in the early 1990s in which mice were immunized against a highly invasive tumor, the adenocarcinoma TA3-Ha, using as an immunogen the Asialo Ovine Submaxillary Mucin (A-OSM) displaying a great number of Tn epitopes. The glycosylated protein elicited protection against the tumor, whereas the unglycosylated mucine did not, thus confirming the specificity for the saccharidic Tn antigen and not for the carrier protein. However, this approach presents two main drawbacks: the carbohydrate contents are usually low in protein conjugates and the construction has a non-defined chemical structure (i.e., the

*Corresponding author. Tel.: +39-02-6448-3453; fax: +39-02-6448-3565; e-mail: laura.cipolla@unimib.it

†Present address: Istituto Oncologico Europeo, Via Ripamonti 435, 20141 Milan, Italy.

‡Present address: Biosearch Italia, Via Lepetit 34, 21040 Gerenzano, Italy.

0968-0896/02/$ - see front matter
PII: S0968-0896(01)00433-3

number of saccharide units linked to the carrier protein). To date, several studies with conjugate cancer vaccines containing natural or synthetic antigens have been reported and many approaches have been explored to increase the immunogenicity of these molecules.[6–10,12] In particular, a novel concept for the antigen construction of antitumour vaccines has been recently developed; according to the reported strategy,[12j] a tumor-associated glycopeptide antigen (MUC1) was combined with a T-cell epitope of tetanus toxin. Moreover, multiple antigen glycopeptides (MAG)[13] have been developed: since the antigens occur on the cell surface as multiple, not single, molecules, it is not surprising that the multiple antigen presentation on synthetic glycoconjugates results in their enhanced immunogenicity.[14]

In this context, we focused our attention on the synthesis of neoglycopeptides **1**–**3** (Fig. 1) as potential small molecular weight antitumor vaccines, constructed by covalently linking a short peptide, as the T-epitope, to the B-epitope, the Tn antigen; in order to achieve increased stability toward in vivo degradation, the two moieties are connected by a *C*-glycosidic bond, in place of the natural *O*-glycosidic linkage.

C-Glycosyl conjugates are, in fact, stable against both chemical and enzymatic degradation and have gained considerable attention in recent years,[15] but their use in biological investigation has been quite rare.[16] In addition, a technique based on the concept of chemoselective ligation[17] has been applied to the site-specific attachment of the sugar moiety to peptides. Chemoselective ligation involves the coupling of two uniquely reactive functional groups with such selectivity that no protecting groups are needed, and can be performed in aqueous solution; to date, different reactions have proven useful for chemoselective ligation.[18] In our case, chemoselectivity was achieved coupling an aminoxy group properly introduced in the peptide backbone with a keto function present on the *C*-glycosyl analogue of the Tn antigen, affording the corresponding oxime.[19]

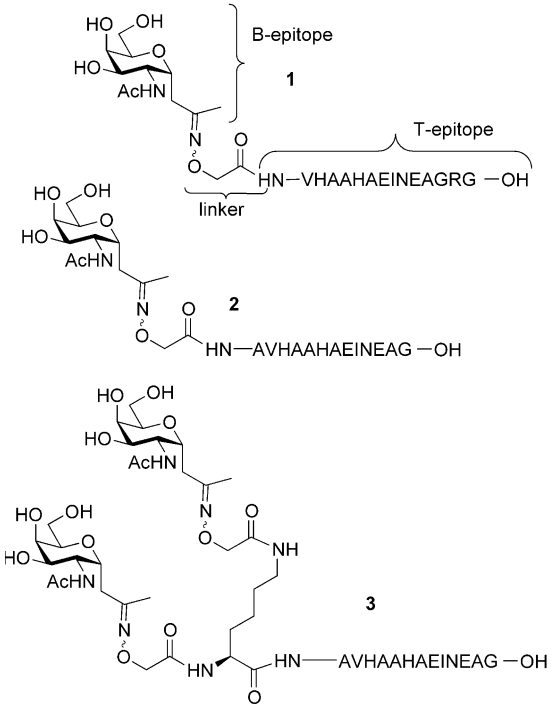


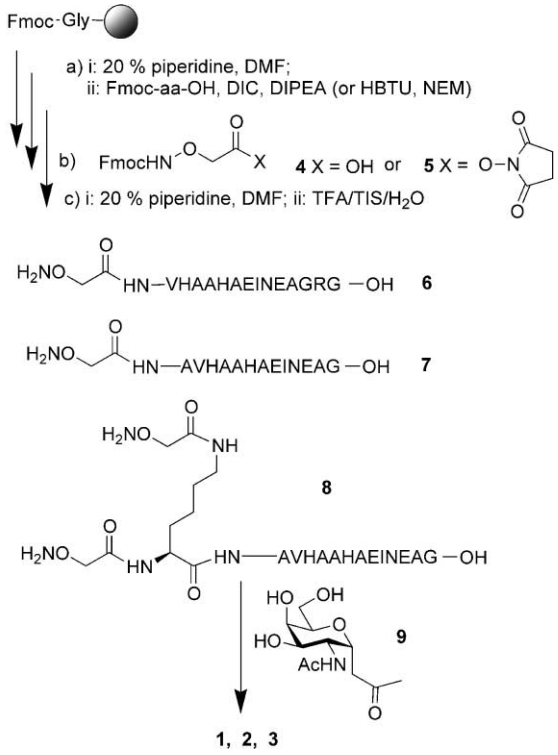
Figure 1. Structure of neoglycopeptides as potential anticancer vaccines.

Results and Discussion

The synthesis of three possible vaccines, **1**–**3**, was performed by a convergent approach based on the chemoselective coupling of α-*C*-glycosyl ketone **9**[20] analogue of *N*-acetyl-galactosamine with peptides **6**–**8** (Scheme 1). The aminooxy acetic acid linker allowed the conjugation of the T-epitope to the B-epitope (Fig. 1). The T-cell epitope of choice is the short peptide fragment 327–339 of ovalbumin ($^{327–339}$OVA) from chicken.[21]

Due to contradictory numbering of the protein, two different sequences were initially synthesized for the attachment of one sugar unit. Fragment peptide **6** derived from previous work by Grey et al. in vitro immunological studies,[22] while peptide **7** corresponded to NCBI database.[21,23]

The peptides were assembled using standard Fmoc protocol for solid-phase peptide synthesis.[24] All amino acids were attached to the resin carrying an N^{α}-Fmoc protective group and acid labile side-chain protecting groups. The attachment of the growing chain to the resin was made by an acid labile linker, in order to cleave the peptides from the solid support and simultaneously hydrolyze the side-chain protecting groups. The



Scheme 1. Synthetic strategy for the preparation of neoglycopeptides **1**–**3**.

last step of the solid-phase synthesis was the introduction of one or two linkers, namely the *N*-Fmoc aminooxy acetic acid **4**, by in situ preactivation or as the corresponding succinate derivative **5**, which introduce in the peptide the required function for the chemoselective ligation with the *C*-glycosidic B-epitope.[20] In order to have a multiple antigen presentation, N^{α},N^{ε}-Fmoc-lysine is added to the N-terminus of the peptide as the last amino acid; deprotection of the two amino function of lysine by standard conditions (piperidine in DMF) and coupling with *N*-Fmoc aminooxy acetic acid allowed the introduction of two sugar moieties into the $^{327–339}$OVA peptide. Conjugation of the α-*C*-glycosyl ketone **9** to the peptides **6**–**8** was performed in acetate buffer at pH 4.5 and monitored by reversed-phase HPLC (RP-HPLC). All the neoglycopeptides presented two isomeric forms in equilibrium, due to the *cis*/*trans* isomerism of the oxime bond, detectable by HPLC analysis and characteristic of oxime-linked glycoconjugates.[19]

Neoglycopeptides **1**–**3** were tested in vitro in an antigen presentation assay either to the T-cell hybridoma BO 97.10,[25] or T cell receptor transgenic T cells from DO 11.10 mice,[25] both specific for the peptide $^{327–339}$OVA in association with the molecule MHC of class II I-A^b on antigen presenting cells (APC). Dendritic cells (DC) are the only APC able to activate naive T cells, that is, T cells that have never seen the antigen.[26] Recognition of peptide–MHC II complexes on the dendritic cell surface results in secretion of interleukin-2 (IL-2) by T-cell hybridomas or in transgenic T-cell proliferation. Interleukin production correlates with the ability of the T-cell receptor (TCR) to recognize the complex MHC–peptide on APC, that is the interleukin produced by the hybridomas reflects the magnitude of the immune response. Secreted IL-2 was then determined in a radioactive proliferation assay by measuring the incorporation of [^{3}H]-thymidine by the IL-2 dependent T-cell clone CTLL.[27] When transgenic T cells were used for the assay, their proliferation was measured directly as [^{3}H]-thymidine incorporation.

Different assays were then performed (Fig. 3). First, we assessed if the additional aminooxy linker would somehow interfere in the recognition between the MHC II molecule and the $^{327–339}$OVA-specific TCR. Thus, the three peptides **6**–**8** were incubated in a dose dependent manner with T-cell hybridomas in 96-well tissue culture plates; as a control the same proliferation assay was performed with synthetic peptide $^{327–339}$OVA. In order to test whether the neoglycopeptides needed to be internalized and processed to be exposed on the cell surface in association with MHC molecules, DC were fixed with glutaraldehyde to block internalization and processing. In other words, if the antigen needed to be processed in order to be presented and recognised by the TCR, it would not be presented correctly with fixed APC. This evidence is clearly represented in Figure 2: the whole ovalbumin needs to be processed and reduced to fragment peptide $^{327–339}$OVA in order to form the peptide–MHC II complex and stimulate T-cells. When DC are fixed, the peptide fragment cannot be formed and there is no production of IL-2.

Hence, in order to check if the aminooxy acetic linker and the additional lysine residue could interfere with the binding to MHC II and with presentation to T cells, peptides **6**–**8** were incubated both with fixed and non-fixed APC. It resulted that for peptides **7** and **8** the

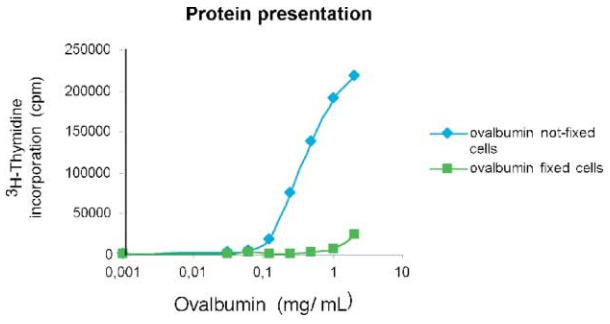


Figure 2. Proliferation of CTLL cells dependent on IL-2 production by hybridoma BO 97.10 incubated with APC and whole ovalbumin.

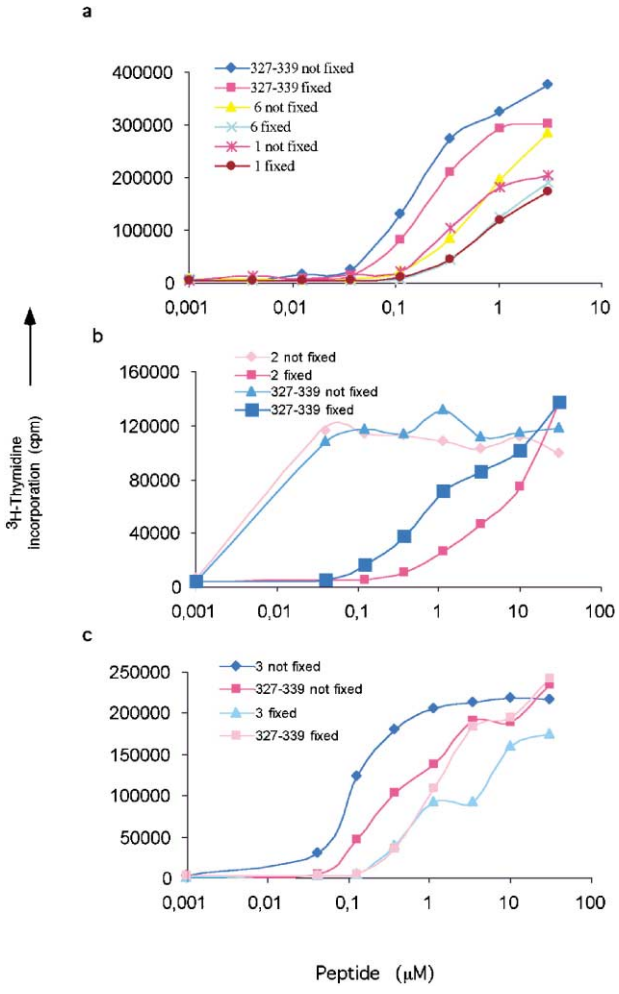


Figure 3. Proliferation of CTLL cells dependent on IL-2 production by hybridoma BO 97.10, incubated with both fixed and non-fixed DC, and the antigen at increasing concentrations: (a) petide **6** and neoglycopeptide **1** showed weaker response compared to the reference peptide $^{327–339}$OVA; (b) neoglycopeptide **2** showed comparable activity than the reference ovalbumin fragment; (c) neoglycopeptide **3** showed a higher response than the $^{327–339}$OVA when using non-fixed cells.

addition of the linker and of the additional lysine at the N-terminus of the sequence did not interfere with the correct presentation of the peptide–MHC II complex to the TCR (data not shown). A different conclusion could be drawn for peptide **6** (Fig. 3a): in this case a lower efficiency in the presentation was observed both with fixed and non-fixed cells. This can be attributed to the 'incorrect' peptide sequence, which is shifted by one amino acid ($^{328-340}$OVA), rather than to the additional groups. Obviously, a similar trend was observed for the neoglycopeptide **1**, which was presented to the TCR less efficiently than the reference fragment peptide $^{327-339}$OVA but similarly to peptide **6** (Fig. 3a). Due to the low efficiency of presentation of neoglycopeptide **1**, the sequence $^{327-339}$OVA was chosen for the synthesis of neoglycopeptides **2** and **3**, bearing one or two B-epitopes, respectively. Identical proliferation tests were then performed with neoglycopeptides **2** and **3** (Fig. 3b and c, respectively). In both cases, the neoglycopeptides could be presented to the TCR also in the absence of internalization and processing, indicating that binding from outside the cells could occur and that the presence of the sugar would not impede TCR–MHC–peptide recognition, as previously observed by Grey[22b] and Unanue.[28]

Interestingly, neoglycopeptide **3**, bearing two sugar moieties, was presented by non-fixed cells with a higher efficiency than the $^{327-339}$OVA peptide at low concentrations (Fig. 3c). This suggested that the glycidic moieties might somehow facilitate either the internalization of the peptide or the activation of T cells may be due to increased costimulation by DC. Activation of DC results in the upregulation of MHC and costimulatory molecules which are necessary for the correct stimulation of T cells. Cross-linking of surface receptors can lead to DC activation. Thus, if the sugar would induce the cross-linking of a putative receptor expressed on the surface of DC, we would expect that only neoglycopeptide **3**, bearing the two sugar moieties, would induce the upregulation of costimulatory molecules on DC. Indeed, as shown in Figure 4, the B7.2 molecule (a prototype of costimulatory molecules expressed by DC) was upregulated only in the presence of neoglycopeptide **3**. A possible artefactual effect due to lipopolysaccharide (LPS) contamination could be excluded because dendritic cells for this experiment were derived from bone-marrow of B10ScCr mice which lack the expression of TLR4, the transducer of cell activation induced by LPS.[29]

In conclusion, conjugation of one or two sugar moieties to the peptidic OVA T-cell epitope does not interfere with the capacity of dendritic cells to present the antigenic peptide to OVA specific T cells. By contrast, the presence of two sugar moieties acts as an adjuvant on DC by upregulating the expression of B7.2 molecule and increasing the presentation of the OVA peptide to T cells. This suggests that the *N*-acetyl galactosamine analogue not only can be used as a B cell epitope, but in multiple copies, it can target the vaccine to dendritic cells by binding and cross-linking its surface receptor. Future perspectives of this work include in vivo studies of induction of immune response and antibody production to the natural tumour associated antigen Tn, after immunization with neoglycopeptide **3** in the presence or absence of exogenous adjuvant. In addition, in order to evaluate the ability of *C*-glycosidic analogue of *N*-acetyl galactosamine to simulate the natural counterpart, protection experiments to the highly aggressive TA3-Ha adenocarcinoma will be performed.

Experimental

General

All solvents were dried over molecular sieves, for at least 24 h prior to use. Thin-layer chromatography (TLC) was performed on Silica Gel 60 F_{254} plates (Merck) with detection with UV light when possible, or charring with a solution containing concd H_2SO_4/EtOH/H_2O in a ratio of 5/45/45. Flash column chromatography was performed on silica gel 230–400 mesh (Merck).

All resins and protected amino acids were purchased from Calbiochem-Novabiochem AG (CH). N^{α}-Fmoc-protected amino acids had the following protecting groups for their side chains: pentamethylchromatin (Pmc) for arginine; *tert*-butyl for glutamic acid; triphenylmethyl (Trt) for histidine and asparagine.

^{1}H and ^{13}C NMR spectra were recorded at 300 MHz on a Brucker AC 300 instrument using $CDCl_3$ as solvent unless otherwise stated. Chemical shifts are reported in ppm downfield from TMS as an internal standard. Mass

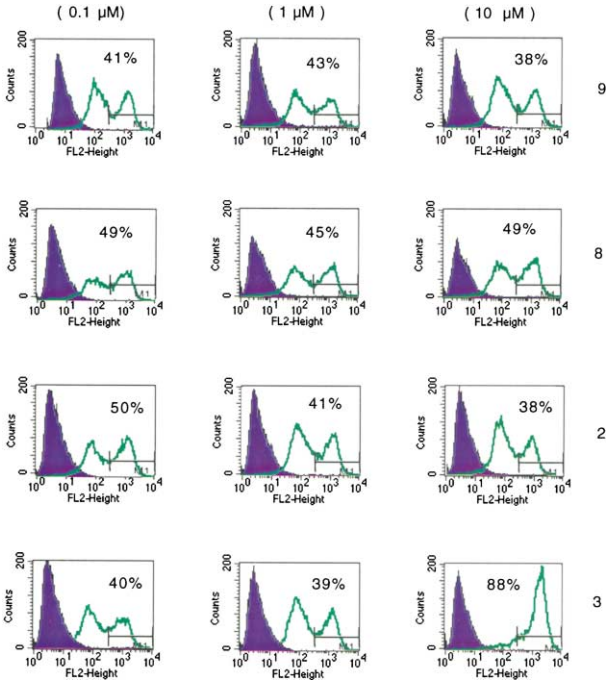


Figure 4. Upregulation of B7.2 molecule after incubation of DC with compounds **9**, **8**, **2**, **3**, respectively. Only neoglycopeptide **3** induced significant upregulation of B7.2. The percentage of B7.2 high cells corresponding to the M1 statistical area is reported.

spectra were recorded on a MALDI2 Kompact Kratos instrument, using gentisic acid (DHB) or α-cyano-4-hydroxycinnamic acid (CHCA) as matrices.

Colorimetric assays. TNBS (2,4,6-trinitrobenzenesulfonic acid) test. [30] Two standard solution are prepared as follows. Solution A: a 10% solution of diisopropylethylamine (DIPEA) in dry DMF; solution B: a 1% solution of TNBS in dry DMF. A small portion of the resin, previously washed with ethanol, is suspended in a mixture of equal amounts of the two solutions. After a few minutes, the presence of unreacted amino groups will be detected by the appeareance of a red colour on the beads.

Bromophenol blue test. [31] A few drops of a 50 μM solution of bromophenol blue in dry DMF is added to a small portion of resin previously washed with DMF; a blue color of the beads indicates the presence of unreacted amino groups.

Purification of peptides was performed by preparative reversed-phase HPLC on a Waters HPLC system equipped with a Merck LiChrosper 100 RP_{18} column (250×10 mm; 10 μm), and eluting with a flow rate of 7 mL/min with eluent A (0.1% TFA in water) and eluent B (0.1% TFA in 90% aq acetonitrile). Analytical HPLC purity checks were performed using a Merck LiChrosper 100 RP_{18} column (250×4 mm; 5 μm), eluting at a flow rate of 1 mL/min and with the same eluent system. Detection was at 215 and 280 nm with an absorbance detector Waters 2487 dual λ.

9-Fluorenylmethoxycarbonyl-aminooxyacetic acid (4). Aminooxy acetic acid hydrochloride (500 mg, 4.57 mmol) was dissolved in an aqueous solution of Na_2CO_3 (1.2 g in 20 mL); the solution was cooled to 0 °C with an ice-bath and then 9-fluorenylmethyl chloroformate (Fmoc-Cl) (1.30 g, 5.02 mmol) in dioxane (10 mL) was added dropwise. The reaction mixture was stirred overnight; the reaction mixture was concentrated, acidified with 5% aqueous HCl and the product precipitated from the solution. 1.1 g (77% yield) of pure **4** were obtained as a white solid. Mp 129–131 °C; ^{1}H NMR (CD_3OD): δ 7.80–7.29 (m, 8H; CH_{arom}), 4.41 (d, J = 7.0 Hz, 2H; $CH_{2\text{-}Fmoc}$), 4.34 (s, 2H; H-2), 4.18 (t, J = 7.0 Hz, 1H; CH_{Fmoc}); MALDI-TOF-MS: calcd for $C_{17}H_{15}NO_5$ 313.3, found m/z $(M+Na)^+$ 336.4, $(M+K)^+$ 352.0.

9-Fluorenylmethoxycarbonyl-aminooxyacetic acid succinic ester (5). Compound **4** (5.29 g, 16.9 mmol) was dissolved in a 1:1 mixture of ethyl acetate/dioxane, and the solution cooled to 0 °C with an ice-bath; *N*-hydroxysuccinimide (2.16 g, 18.8 mmol) and dicyclohexylcarbodiimide (3.88 g, 18.8 mmol) were sequenctially added to the solution. After 24 h, the precipitate was filtered off, and the solvent evaporated to dryness under reduced pressure. The residue was then dissolved in ethyl acetate, washed with 5% aq $NaHCO_3$, the organic layer dried over Na_2SO_4, filtered and evaporated. The active ester **5** was obtained in 93% yield (6.42 g) as a white solid. Mp 95–97 °C; ^{1}H NMR: δ 8.29 (s, 1H; NH), 7.80–7.27 (m, 8H; CH_{arom}), 4.72 (s, 2H; H-2), 4.47 (d, J = 6.9 Hz, 2H; $CH_{2\text{-}Fmoc}$), 4.22 (t, J = 6.9 Hz, 1H; CH_{Fmoc}), 2.82 (s, 4H; CH_2CON); MALDI-TOF-MS: calcd for $C_{21}H_{18}N_2O_7$ 410.4, found m/z $(M+Na)^+$ 433.6, $(M+K)^+$ 449.7.

Peptide 6. Synthesis. The synthesis was carried out using 1 g of a Wang resin (100–200 mesh, maximum loading 0.76 mmol/g). The first amino acid (N^{α}-Fmoc-Gly-OH) was coupled to the resin upon activation of the carboxylic group as 1-benzotriazolyl ester prepared in situ by reaction of the amino acid (684 mg, 2.3 mmol, 3 equiv), 1-hydroxybenzotriazole (HOBt, 311 mg, 2.3 mmol, 3 equiv) catalytic 4-dimethylaminopyridine (DMAP, 56 mg, 0.46 mmol, 0.2 equiv), 1,3-diisopropylcarbodiimide (DIC, 358 μL, 2.3 mmol, 3 equiv) in dry DMF (15 mL). After 30 min, the solution was added to the resin, which was mechanically stirred for 16 h. Resin was washed with DMF and dichloromethane between each coupling and deprotection step. After the first coupling, a capping step of possibly unreacted hydroxyl functions on the resin was performed; the resin was reacted with a 10% solution of acetic anhydride in dry DMF for 2 h (10 mL); afterwards, the resin was washed as usual. N^{α}-Fmoc deprotection of the peptide resin was performed by treatment with 20% piperidine in DMF (3×10 min). All subsequent N^{α}-Fmoc amino acids (2.3 mmol, 3 equiv) were coupled after preactivation with HOBt (311 mg, 2.3 mmol, 3 equiv), DIC (358 μL, 2.3 mmol, 3 equiv), DIPEA (400 μL, 2.3 mmol, 3 equiv), in dry DMF (15 mL) for 30 min. As the last step on solid-phase 9-fluorenylmethoxycarbonyl-aminooxyacetic acid succinic ester **5** (944 mg, 2.3 mmol, 3 equiv) was added to the peptide resin dissolved in dry DMF (10 mL), using DIPEA (400 μL, 2.3 mmol, 3 equiv), as the base. The completeness of all reactions was monitored by TNBS test.

Cleavage. Peptide **6** was cleaved from the resin by incubation with a 95:2.5:2.5 mixture of trifluoroacetic acid (TFA)/triisopropylsilane[32]/H_2O (10 mL). After 2 h, the resin was removed by filtration and washed carefully with the cleavage mixture (2×5 mL). The combined filtrates were concentrated, then addition of cold diethyl ether (10 mL) allowed the precipitation of the crude peptide **6**, which finally was filtered and dried.

Purification. Purification of the crude by preparative-scale HPLC (eluent: linear gradient 10→50% eluent B in eluent A over 40 min), afforded peptide **6** in 10% yield. The purity was checked by analytical HPLC (eluent: linear gradient 0→100% eluent B in eluent A; analysis time 30 min, t_R = 12.7 min). MALDI-TOF-MS: calcd 1504, found m/z $(M+H)^+$ 1505.

Neoglycopeptide 1. Peptide **6** (50 mg, 0.033 mmol) was dissolved in a buffer solution at pH = 4.5 (1.5 mL) prepared adjusting a 0.1 M solution of sodium acetate to the desired pH with glacial acetic acid. The *C*-glycoside **9**[20] (11 mg, 0.042 mmol) dissolved in water was then

added to the aminooxy peptide. The reaction mixture was allowed to stir for 90 min, and monitored by reversed-phase analytical HPLC (eluent: linear gradient 10→50% eluent B in eluent A over 40 min; $t_R = 15.2$ and 15.5 min). The solution was then freeze-dried and the crude purified by preparative-scale HPLC (eluent: linear gradient 15→25% eluent B in eluent A over 40 min). 19 mg of neoglycopeptide **1** were obtained, corresponding to 33% yield, as a mixture of *E*/*Z* isomeric forms. MALDI-TOF-MS: calcd 1748 found *m*/*z* $(M-H_2O+Na)^+$ 1752; amino acid analysis: Ala 4.02 (4), Arg 0.99 (1), Asn 1.01 (1), Gly 2.01 (2), Glu 1.98 (2), His 2.03 (2), Ile 0.98 (1), Val 1.00 (1).

Peptide 7. Synthesis. The synthesis was carried out on 215 mg of HMPA-Novagel® resin (100–200 mesh, maximum loading 0.67 mmol/g). The first amino acid (N^{α}-Fmoc-Gly-OH, 128 mg, 0.43 mmol, 3 equiv) was coupled to the resin upon activation with 1-(mesitylsulfonyl)-3-nitro-1,2,4-triazole (MSNT, 127 mg, 0.43 mmol, 3 equiv) in the presence of 1-methylimidazole (26 μL, 0.32 mmol, 2.25 equiv) in dry CH_2Cl_2 (5 mL) for 45 min.[33] The coupling was repeated a second time with the same quantities of reactants and reaction time. Resin was washed with dichloromethane and DMF. After the first coupling a capping step of possibly unreacted hydroxyl functions on the resin was performed; the resin was reacted with a 10% solution of acetic anhydride in dry DMF for 2 h (5 mL); afterwards the resin was washed with DMF. N^{α}-Fmoc deprotection of the peptide resin was performed by treatment with 20% piperidine in DMF (2 + 16 min). Resin was washed with DMF (6×2 min) between each coupling and deprotection step. All subsequent N^{α}-Fmoc amino acids (0.43 mmol, 3 equiv) were coupled with *O*-(benzotriazol-1-yl)-N,N,N′,N′-tetramethyluronium hexafluorophosphate (HBTU, 153 mg, 0.40 mmol, 2.8 equiv), and *N*-ethylmorpholine (NEM, 82 μL, 0.65 mmol, 4.5 equiv) The final coupling with *N*-Fmoc protected aminooxyacetic acid **4** was carried out as with the other amino acids. The N-terminal Fmoc protecting group was cleaved by treatment with 20% piperidine, the resin was washed once more with DMF (6×). The completeness of all reactions was monitored by the bromophenol blue test.

Cleavage. Peptide **7** was cleaved from the resin by incubation with a 95:2.5:2.5 mixture of TFA/triisopropylsilane/H_2O (5 mL). After 2 h, the resin was removed by filtration and washed carefully with the cleavage mixture (2×5 mL). The combined filtrates were concentrated, then addition of cold diethyl ether (10 mL) allowed the precipitation of the crude peptide **7**, which finally was filtered and dried.

Purification. Purification of the crude by preparative-scale HPLC (eluent: linear gradient 10→40% eluent B in eluent A over 50 min), afforded peptide **7** in 22% yield. The purity was checked by analytical HPLC (eluent: linear gradient 10→40% eluent B in eluent A; analysis time 50 min; $t_R = 16.3$ min). MALDI-TOF-MS: calcd 1362, found *m*/*z* $(M+Na)^+$ 1386; $(M+K)^+$ 1401.

Neoglycopeptide 2. Peptide **7** (4.0 mg, 0.003 mmol) was dissolved in a buffer solution at pH 4.5 (300 μL). The *C*-glycoside **9** (1.0 mg, 0.004 mmol, 1.3 equiv) dissolved in water (300 μL) was then added to the aminooxy peptide. The reaction mixture was stirred for 30 min, and monitored by reversed-phase analytical HPLC (eluent: linear gradient 10→40% eluent B in eluent A over 50 min; $t_R = 19.1$ and 19.6 min). The solution was then freeze-dried and the crude purified by preparative HPLC (eluent: linear gradient 10→40% eluent B in eluent A over 50 min). 3.4 mg of neoglycopeptide **2** (71% yield) were obtained as a mixture of *E*/*Z* isomeric forms. MALDI-TOF-MS: calcd 1605, found *m*/*z* $(M+Na)^+$ 1629; amino acid analysis: Ala 5.03 (5), Asn 0.99 (1), Gly 1.02 (1), Glu 1.99 (2), His 2.01 (2), Ile 0.98 (1), Val 1.02 (1).

Peptide 8. Synthesis. The synthesis was carried out on 150 mg of HMPA-Novagel® resin (100–200 mesh, maximum loading 0.67 mmol/g). The first amino acid (N^{α}-Fmoc-Gly-OH, 0.30 mmol, 89 mg, 3 equiv) was coupled to the resin upon activation with MSNT (89 mg, 0.30 mmol, 3 equiv) in the presence of 1-methylimidazole (18 μL, 0.22 mmol, 2.25 equiv) in dry CH_2Cl_2 (4 mL) for 45 min. The coupling was repeated a second time with the same quantities of reactants and reaction time. Resin was washed with dichloromethane and DMF. After the first coupling a capping step with a 10% solution of acetic anhydride in dry DMF for 2 h (5 mL) was performed; afterwards the resin was washed with DMF. N^{α}-Fmoc deprotection of the peptide resin was accomplished by treatment with 20% piperidine in DMF (2 + 16 min). Resin was washed with DMF (6×2 min) between each coupling and deprotection step. All subsequent N^{α}-Fmoc amino acids (0.30 mmol, 3 equiv) were coupled with HBTU (106 mg, 0.28 mmol, 2.8 equiv), and NEM (57 μL, 0.45 mmol, 4.5 equiv). The coupling with N^{α}-Fmoc protected aminooxyacetic acid **4** to both the α and ε amino groups of terminal lysine was carried out as with the other amino acids, doubling all the reactants (6 equiv of **4**, 9 equiv of NEM, 5.6 equiv of HBTU). The two N-terminal Fmoc protecting groups were cleaved by treatment with 20% piperidine, the resin was washed once more with DMF (6×). The completeness of all reactions was monitored by the bromophenol blue test.

Cleavage. Peptide **8** was cleaved from the resin by incubation with a 95:2.5:2.5 mixture of TFA/triisopropylsilane/H_2O (5 mL). After 2 h, the resin was removed by filtration and washed carefully with the cleavage mixture (2×5 mL). The combined filtrates were concentrated, then addition of cold diethyl ether (10 mL) allowed the precipitation of the crude peptide **8**, which was finally filtered and dried.

Purification. Purification of the crude by preparative-scale HPLC (eluent: linear gradient 10→40% eluent B in eluent A over 50 min), afforded peptide **8** in 9% yield. The purity was checked by analytical HPLC (eluent: linear gradient 10→40% eluent B in eluent A; analysis time 50 min, $t_R = 12.3$ min). MALDI-TOF-MS: calcd

1563, found m/z $(M+H)^+$ 1565; $(M+Na)^+$ 1587; $(M+K)^+$ 1604.

Neoglycopeptide 3. Peptide **8** (3.0 mg, 0.002 mmol) was dissolved in a buffer solution at pH 4.5 (300 μL). The *C*-glycoside **9** (1.4 mg, 0.005 mmol, 2.6 equiv) dissolved in water (300 μL) was then added to the aminooxy peptide. The reaction mixture was stirred for 40 min, and monitored by reversed-phase analytical HPLC (eluent: linear gradient 10→40% eluent B in eluent A over 50 min; $t_R = 21.0$ and 25.5 min). The solution was then freeze-dried and the crude purified by preparative HPLC (eluent: linear gradient 10→40% eluent B in eluent A over 50 min). 2.5 mg of neoglycopeptide **3** (62% yield) were obtained as a mixture of *E*/*Z* isomeric forms. MALDI-TOF-MS: calcd 2050 found m/z $(M+H)^+$ 2052; amino acid analysis: Ala 4.99 (5), Asn 1.00 (1), Gly 1.03 (1), Glu 2.02 (2), His 1.98 (2), Ile 1.01 (1), Lys 1.02 (1), Val 0.99 (1).

Determination of T-cell hybridoma response. The response of T-cell hybridoma BO 97.10, specific for the peptide $^{327-339}$OVA in association with the molecule MHC of class II I-A^b was measured as secreted IL-2 upon incubation of the hybridoma with antigen presenting cells (APC). A growth factor-dependent long term culture dendritic cells (DC) line was used (D1) as a source of APC.[34] Secreted IL-2 was determined by a standard assay with a IL-2 dependent T-cell clone CTLL. Hybridomas BO 97.10 were suspended in IMDM 10% fetal bovine serum (GIBCO), 100 IU/mL penicillin, 100 mg/mL streptomycin, 2 mM L-glutamine (purchased by Sigma) and 50 μM 2-mercaptoethanol (1×10^6 cells/mL). D1 cells were suspended in IMDM 5% fetal calf serum (2×10^5 cells/mL) or in 0.001% glutaraldehyde in PBS for 1 min to fix the cells. D1 cells were co-cultured (1×10^4 cells/well) with BO 97.10 (5×10^4 cells/well) and with different concentrations of antigen (whole ovalbumin, $^{327-339}$OVA peptide, peptides **6**–**8** and neoglycopeptides **1**–**3**) in 96-well tissue culture plates (Corning). After 24 h, supernatants were collected and added to murine CTLL line (1×10^4 CTLL in IMDM 5% FCS). The CTLL cultures were incubated for 24 h, and then pulsed with 1 μCi [^{3}H]thymidine/well for an additional 6 h (1 μCi/mL Amersham). [^{3}H]thymidine incorporation was measured by using a cell harvester (Wallac TOMTEC).

Lymphocyte proliferation assay. Spleen cells (3×10^6 cells/well) from TCR-OVA DO11.10 mice were resuspended in IMDM containing 5% FCS and were incubated in a 96-well tissue culture plates (Corning) with different concentrations of the antigen (whole ovalbumin, $^{327-339}$OVA peptide, peptides **6**–**8** and neoglycopeptides **1**–**3**). After 24 h the cultures were pulsed with 1 μCi [^{3}H]thymidine/well for 3 days. Cells were then harvested and radioactive incorporation was determined by scintillation counting. The same assay was performed after purification of CD4+ T lymphocytes by positive selection with α-CD4 antibodies coupled to magnetic beads, using MiniMACS separating column (Miltenyi Biotec, Auburn, CA, USA).

Phenotypical activation of DC. Bone marrow derived DC (BMDC) were generated from single cell suspension of marrow from femurs of C57BL/10ScCr mice, which lack the gene expressing for TLR4 . BM cells were split every 5 days with PBS to detach only loosely adherent cells. After 15–20 days of culture in DC medium, homogeneity of DC was evaluated by cytofluorimetry. Only cultures containing more than 90% cells expressing intermediate levels and less than 10% cells expressing high levels of MHC class II (I-Ab) and B7.2 were used for the experiments. BMDC were incubated with concentrations of peptides ranging between 0.1 and 10 μM for 24 h in IMDM medium containing 30% GM-CSF conditioned medium.[35] Cells were detached in PBS 2 mM EDTA, stained with anti B7.2 antibody (CD86/B7.2: GL1, Pharmingen, BD) and analyzed by cytofluorimetry (FACS) using the Cell Quest software (BD).

Acknowledgements

We thank the Ministero dell'Università e della Ricerca Scientifica e Tecnologica (MURST) for the financial support.

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